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Isolation and Purification of Proteins Induced by Vitamin K Absence

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Our interest in the influence of coumarin congeners on the spectrum of coagulation factors in man dates from about 1962.

It arose from the observation that in the hands of our clinicians oral anticoagulation could serve as an efficient tool in the secondary prevention of coronary infarction, but only under the very strict condition that a constant low level of Factors II, VII, IX, and X could be maintained. The problems associated with keeping patients safely between 12 and 20% of their normal prothrombin level required a thorough investigation of the tests of the prothrombin-time type. All of these tests proved to give different results. This was mainly because thromboplastins differed in sensitivity to a competitive inhibitor of blood coagulation that could be demonstrated in the plasma, both during oral anticoagulation and real vitamin K deficiency.

In 1963, we were the first to postulate that in vitamin K deficiency not only is the synthesis of certain coagulation factors inhibited, but, at the same time, the appearance is induced of a protein acting as a competitive inhibitor of prothrombinase formation. We called this protein PIVKA (Protein Induced by Vitamin K Absence or Antagonists). At that time, it was taking a long but carefully aimed shot to postulate that the inhibitor is a precursor of prothrombin, appearing in the plasma because the absence of vitamin K blocks a postribosomal step in prothrombin formation. We must say that we are very happy to see that subsequent research in our own, as well as in many other laboratories, has fully justified the idea.

At the time we felt like an astronomer, whose theoretical speculations and calculations indicate the existence of another planet, but who has not yet been able to find it with his telescope.

Our next aim, therefore, was to demonstrate the existence of PIVKA by chemical means and to purify it.

As a working hypothesis, we always have favoured the idea that coagulation Factors II, VII, IX, and X are four different polypeptide chains. If our hypo-

thesis on the origin of PIVKA was right, we could expect to find four different PIVKAs in anticoagulated individuals; one for each of the coagulation factors sensitive to vitamin K. We called them PIVKA-II, PIVKA-VII, PIVKA-IX, and PIVKA-X.

Human PIVKA-II was the first to surrender its elusive character. In 1966, using antibodies specific against human Factor II, Josso demonstrated that coumarin-treated individuals have two populations of molecules which precipitate with this antibody. This work was later repeated by Niléhn and Ganroth and by Brozovic. Recently, Stenflo isolated PIVKA-II from the plasma of coumarin-treated cows, and you have just heard Dr. Malhotra describing similar experiments.

Obviously, the next step was to try and find the other PIVKAs. Evidence for their existence was not impressive, the only reliable indication being that Veltkamp of our group found that an acquired antibody to human Factor IX was neutralized by vitamin K deficient plasma to a far greater extent than could be accounted for by the Factor IX activity assessed in a Factor IX assay. Another indication was that the PIVKA inhibiting prothrombin-time competed with Factor X. It was, therefore, more likely than not to be a Factor X analogue. But because of the similarity between Factors X and II, it would hardly be surprising if a precursor of either one could act as a competitive inhibitor.

We first prepared monospecific antibodies against the bovine coagulation Factors II, IX, and X, but did not succeed in producing antibodies against Factor VII.

Most of you will agree that preparing the pure factor and producing monospecific antibodies, although they can be reported quickly, form the most difficult and time consuming part of the job. Actually, it took us five years. We then anticoagulated a cow – as you know cows abound in Holland, and our clinicians are great in anticoagulation. This was, therefore, a relatively easy task.

On another occasion I will perhaps be able to tell you about the interesting conclusions one can draw from these observations concerning the *in vivo* synthesis of coagulation factors; here I will stick to my subject and restrict myself to what can be demonstrated in the plasma of such cows.

To start with, quantitative immunoprecipitation curves showed that with only a fraction of the normal coagulant activity present in the samples of anticoagulated plasma, the amounts of material precipitated by a specific antibody in blood of anticoagulated cows and blood from normal cows, were still about equal.

Coagulant activity of the supernatant fluid disappeared in the zone of antibody excess, indicating that it was indeed the specific antibody that caused precipitation.

Furthermore, it could be seen that there must be some difference in precipitation equilibrium between each of the normal factors and their corresponding PIVKAs. Although this made exact quantitation in the next series of experiments impossible, the precipitation equilibria were close enough to permit a semiquantitative analysis.

By means of one-dimensional (Laurell) electrophoresis, an excess of precipitable material relative to the procoagulant activity could be demonstrated in the plasma of anticoagulated cows. The qualitative results were even more interesting. The $Al(OH)_3$ adsorbed plasma of anticoagulated cows retained precipitating material that disappeared from normal plasma treated in the same way. This held for all of the three factors investigated.

In a third series of experiments this property was explored further. The results showed that the PIVKAs were adsorbable only to high concentrations of $Al(OH)_3$, whereas the normal fractions disappeared at much lower concentrations of the adsorbant.

The fourth investigation demonstrated the existence of two populations of each of the factors under consideration. This study was carried out by two-dimensional Laurell electrophoresis, as first done by Josso for human Factor II.

The basic idea was that the presence of Ca^{++} ions slows down the electrophoretic mobility of the normal factor, but does not affect the PIVKAs. The PIVKAs, therefore, presumably lack the ability to bind Ca^{++}. Camelback pictures were obtained from coumarin plasmas with antibodies against each of the Factors II, IX, and X.

The absence of crossing downslopes is one of the indications that a different mobility of the same antigenic determinant caused this picture.

Eventually, we used an immunoadsorbent column to obtain PIVKA-X and PIVKA-IX in a pure state. The column was prepared by fixing the antibodies in a polyacrylamide gel. This type of column also retained Factor X material from coumarin plasma and from $Al(OH)_3$ adsorbed coumarin plasma, but not from $Al(OH)_3$ adsorbed normal plasma. So it presumably purified PIVKA.

Purified X could be activated by RVV, whereas PIVKA-X could not. PIVKA-X was shown not to inhibit the activation of normal X by RVV either. Using these two preparations we were able to resolve the camelback Laurell picture in two components; the combined preparations yield a camelback picture again.

I think it may be said that it has been established beyond reasonable doubt that not only PIVKA-II, but also the PIVKAs IX and X do exist in the anticoagulated cow.